academicJournals

Vol. 14(31), pp. 2472-2477, 5 August, 2015
DOI: 10.5897/AJB2015.14510
Article Number: 6CA6B2554561
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Characterization of immobilized post-carbohydrate meal salivary α-amylase

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Received 17 February, 2015; Accepted 29 June, 2015

Saliva containing amylase was collected from an individual 5 min after a carbohydrate meal and filtered using a dialysis bag to remove starch particles. The filtered saliva was immobilized on calcium alginate beads. The effect of experimental parameters like pH, temperature and substrate concentration on the activity of the immobilized post-carbohydrate meal salivary α-amylase was determined. The immobilized salivary α-amylase had an optimum activity at temperature 40°C and pH 7.0. The activation energy (Ea) as obtained from the Arrhenius plot was 31.4 kJ/Mol. The kinetic parameters Km and Vmax of the immobilized α-amylase were found to be 1.6 mg/ml and 16.4 µmol/min, respectively; this was compared to that of free salivary α-amylase (Km = 0.0048 mg/ml) and α-amylases from fungi and bacteria sources. Immobilization tends to increase the Km of the immobilized enzyme, indicating a low affinity for substrate, however the enzymes Km was lower than that of some microbial α-amylases. The results obtained from the characterization of immobilized post-carbohydrate meal salivary α-amylase in this study show that immobilization had no significant effect on the enzyme and compared to kinetic parameters of microbial α-amylase, immobilized salivary α-amylase may not be of significant benefit as alternative source of α-amylase in the industrial bioprocesses.

Key words: Enzyme activity, carbohydrate, immobilized enzyme, industrial bioprocess, kinetics, salivary α-amylase.

INTRODUCTION

Alpha amylases have been on increasing demand for various industries, due to their applications in the production of wide array of products, ranging from conversion of starch to sugar syrups, textile, paper, brewing, baking, distilling industries, preparation of digestive aids, production of cakes, and the production of cyclodextrins for the pharmaceutical industry (Sivaramakrishan et al., 2006; Gupta et al., 2003; Reddy et al., 2003). These enzymes account for about 30% of the world's enzyme production (Chi et al., 2009; Van der Maarel et al., 2002). Hence, there is enormous interest in developing alpha amylases with better properties. Alpha amylases (endo-1, 4-α-D-glucanglucanohydrolase EC 3.2.1.1) are extracellular endo enzymes that randomly

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Abbreviations: Ea, Activation energy.

cleave α-1,4 linkages between adjacent glucose units in the linear amylose chain and ultimately generate glucose, maltose and maltotriose units (Uma and Nasrin, 2013). Most of the α- amylases are metallo-enzymes, which require calcium ions (Ca^{2+}) for their activity, structural integrity and stability. The α-amylase family can roughly be divided into two groups: the starch hydrolyzing enzymes and the starch modifying, or transglycosylating enzymes (Sobukola and Aboderin, 2012). The enzymatic hydrolysis is preferred to acid hydrolysis in starch processing industry due to a number of advantages such as specificity of the reaction, stability of the generated products, lower energy requirements and elimination of neutralization steps (Satyanarayana et al., 2005).

The human buccal cavity (mouth) can be likened to a template of industrial reactor whose salivary amylase may possess some industrial applications when characterized. Although, the quantity of salivary amylase may be small in the mouth, it may possess better kinetic parameters which could be genetically engineered to complement microbial sources of the amylase. The human salivary α-amylases initiate the chemical process of digestion in the mouth and like amylases from other sources; break down large insoluble starch molecules into soluble starches producing successively smaller starch molecules and ultimately maltose. They act only on linear α-1,4-glycosidic bonds, and hence depend on other enzymes like β-amylase and glucosidase for complete hydrolysis of branched products (Sivaramakrishnan et al., 2006; Robert et al., 2006; Uma and Nasrin, 2013).

Research has shown that the enzyme is optimally active at temperature of 37°C and pH range of 6.7 to 7.0 (Vijayaraghavan et al., 2011). However, the enzyme is inactivated in the stomach by gastric juice at pH 3.3 (Agamemnon and Stefan, 2003). The enzyme is a metalloenzyme and thus requires the presence of metallic ions like calcium and sodium for effective catalytic activity (Siddhartha et al., 2013). The aim of this study was to examine the kinetic property of the immobilized human salivary α-amylase as a possible alternative source of industrial enzyme.

MATERIALS AND METHODS

Collection of salivary α-amylase

Saliva (5 ml) was collected from ten (10) individuals 5 min after a carbohydrate meal. The saliva samples were then filtered using a porous material to remove starch particles. About 1.5 g of sodium alginate was dissolved in 50 ml of distilled water and autoclaved at 121°C for 15 min after cooling to room temperature. 4.5 ml of the crude enzyme sample was added and mixed. The mixture was then allowed to stand for 10 min. The enzyme-alginate mixture was carefully pumped through sterile syringes drop wise into beaker containing 250 ml of sterile 0.12 g calcium chloride in order for the mixture to form beads. The beads were kept in solution for 1 h at 4°C to ensure complete precipitation according to a modified method of James (1992).

Determination of the number of beads of immobilized salivary α-amylase with highest activity

The number of beads that gave the maximum activity with time was determined. Six (6) test tubes were used. The first test tube contained 1 bead + 5 ml of 1% starch + 1 ml of NaCl. The second test tube contained 2 beads + 5 ml of 1% starch + 1 ml of NaCl until the sixth test tube with 6 beads. It was found that 2 beads of size 2 mm yielded the maximum activity and hence was used to characterize the immobilized enzyme. Activity of the immobilized salivary α-amylase was defined as the amount of glucose produced in mM/min at a given temperature when the substrate is hydrolyzed by the enzyme.

Effect of substrate concentration, temperature and pH on α-amylase activity

Starch concentration for optimum extracellular amylase activity varied from 5.0 to 30 mg/ml in 0.1 M potassium phosphate buffer (pH 7.5). Kinetic data were transformed into Lineweaver–Burk plots with graph pad prismprogram (version 5.0). The Km value was calculated by slope of the curve. Extracellular α-amylase activity was also performed at different temperatures ranging from 20 to 70°C. Effect of pH was assayed using 0.1 M pH buffer solutions ranging from pH 4.5 to 9 in increments of one pH unit, (Note that phosphate buffer is only good for pH = 4.5 to 9 due to the dissociation constant). All enzyme assay measure either the consumption of substrate or production of products over time. Initial rate experiments were used to measure the amylase activity. When an enzyme is mixed with a large excess of substrate, the enzyme-substrate intermediates build up in a fast initial transient. Then the reaction achieves a steady-state kinetics in which enzyme substrate intermediates remains approximately constant over time and the reaction rate changes relatively slow. Rates are measured for a short period after the attainment of a quasi-steady state, typically by monitoring the accumulation of products with time. Because the measurements are carried for a short period of time also large excess substrate, the assumption that the amount of free substrate is approximately equal to the amount of initial substrate is usually made. The enzyme activity is the measure of the quantity of active enzyme present. There are two ways to measure enzyme activity; monitoring the disappearance of substrate or the appearance of product. Measuring the appearance of the product is usually accurate because detecting small changes in [P] ([P]=0) is easier to measure than detecting small changes in [S].

Amylase activity = moles of substrate per unit time = rate x reaction volume.

RESULTS

The result from the plot of immobilized post carbohydrate meal salivary α-amylase activity against temperatures values ranging from 25 to 70°C showed that temperature effect on the immobilized salivary α-amylase was optima at 40°C (Figure 1). In Table 1, optimal temperatures and activities of immobilized human salivary α-amylase samples were compared with known microbial α-amylases. The result showed that the salivary amylase had significantly ($p<0.05$) lowered optimum temperature than the microbial sources. The pH effect on the immobilized enzyme was also determined within pH values range of 4.5 to 9.0; the enzyme was found to be

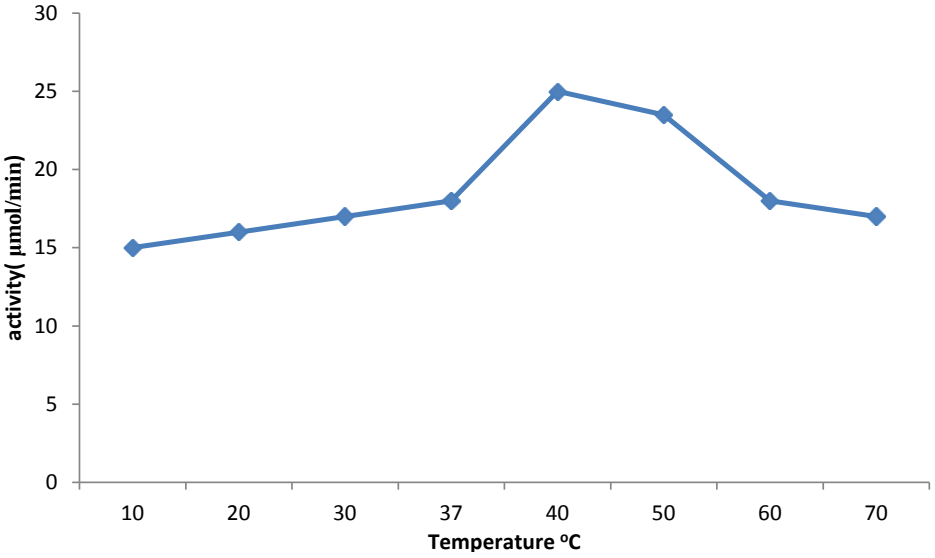

Figure 1. Effect of temperature on human immobilized salivary α-amylase activity showing optimum temperature of 40°C.

Table 1. Comparison between residual activity of human salivary α-amylase and microbial α-amylases at different temperatures.

Organism	Temperature range (°C)	Residual activity (%)	Temperature Optimum (°C)	References
Human salivary (immobilized)	20 - 70	25 (20 min)	40	This study
Bacillus sp. PS1-3	65 - 100	50 (80°C for 2.5 h)	70	Goyal et al. (2005)
Pyrococcus furiosus	80 - 100	50 (98°C for 13 h)	100	Vieille and Zeikus (2001)
Aspergillus tamarii	50 - 60	90 (65°C for 3 h)	55	Moreira et al. (2004)
Lactobacillus manihotivorans	50 - 70	70(80°C for 2.5 h)	55	Aguilar et al. (2000)

optimally active at pH 7.0 (Figure 2); this was compared with optimal pH values of fungal and bacteria α-amylases as shown in Table 2. The activation energy of the immobilized enzyme obtained from an Arrhenius plot was 1.4 kJ/mol (Figure 3). The Km and Vmax of the enzyme were 1.6 mg/ml and 16.4 µmol/min, respectively, as obtained from a Line weaver-Bulk plot (Figure 4).

DISCUSSION

In spite of the wide distribution of α-amylases from fungal and bacterial sources, the diversity of its industrial application creates the need to search for novel α-amylases with novel and improved properties. The rate of hydrolysis of starch by α-amylase depends on many process conditions such as temperature, pH, nature of substrate, substrate concentration, enzyme concentration, presence of Ca^{2+} and other stabilizing agents. α -amylases with properties suitable for industrial conditions and applications have to be appropriately selected based on their high demand. Results obtained from this research work showed that the immobilized salivary α-amylase activity increased as temperature was increased from 20°C. This initial rise in temperature could be due to the increase in probability of effective collision between the reactants as a result of an increase in their average kinetic energy (Okoye et al., 2013). The optimal temperature was obtained at 40°C (Figure 1), followed by a sudden decrease in the activity with further increase in temperature. It is desirable that α-amylases be active at high temperatures of gelatinization (100 to 110°C) and liquefaction (80 to 90°C) to economize the industrial bioprocess; α-amylases from microbial sources seems to be more thermostable (Table 1) than the immobilized salivary α-amylase as observed in this research work (Haki and Rakshit, 2003).

Similarly, the results also showed that the activity of the immobilized enzyme was affected by changes in pH. As the pH of the medium was increased, the activity of the enzyme also increased up to an optimum value of 7.0 (Figure 2). Further increase in pH resulted to a fall in activity and this could possibly be due to ionization of amino-acid side chains that are involved in the catalytic

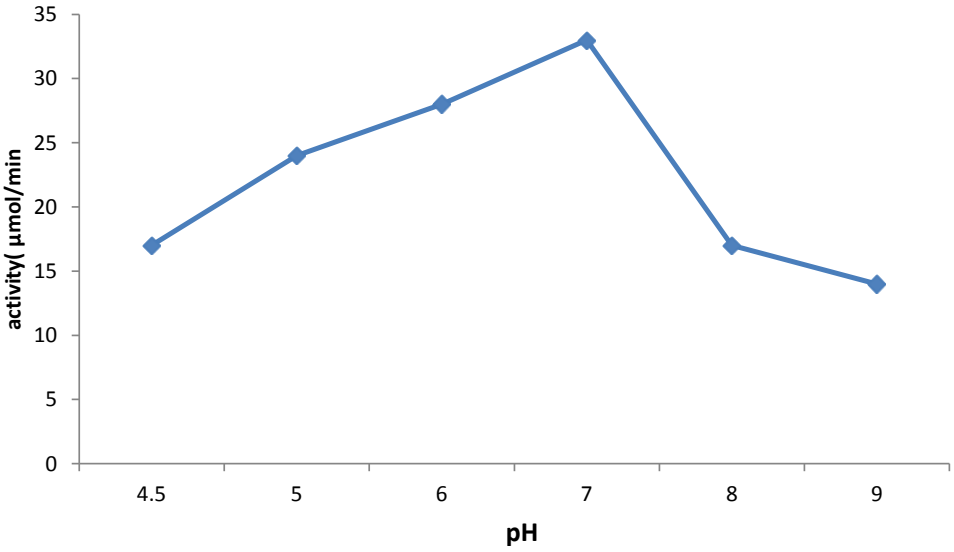

Figure 2. Effect of pH on the activity of human immobilized salivary α-amylase showing optimum pH at 7.

Table 2. Comparison between residual activity of human salivary α-amylase and microbial α-amylases at different pH.

Organism	pH range	Residual activity (%)	pH optimum	Reference
Human saliva (immobilized)	4.5 - 9	32 (20 min)	7.0	This study
Bacillus sp. PS-7	5.0 - 8	96 (pH = 5.0 for 90 min)	6.5	Sodhi et al. (2005)
Bacillus sp.PS1-3	5.0 - 5.5	55 (pH = 10 for 15 h)	5.5	Goyal et al. (2005)
Bacillus sp. *ANT-6*	9 - 13	90 (pH 2.0 for 30 min)	10.5	Burhan et al. (2004)

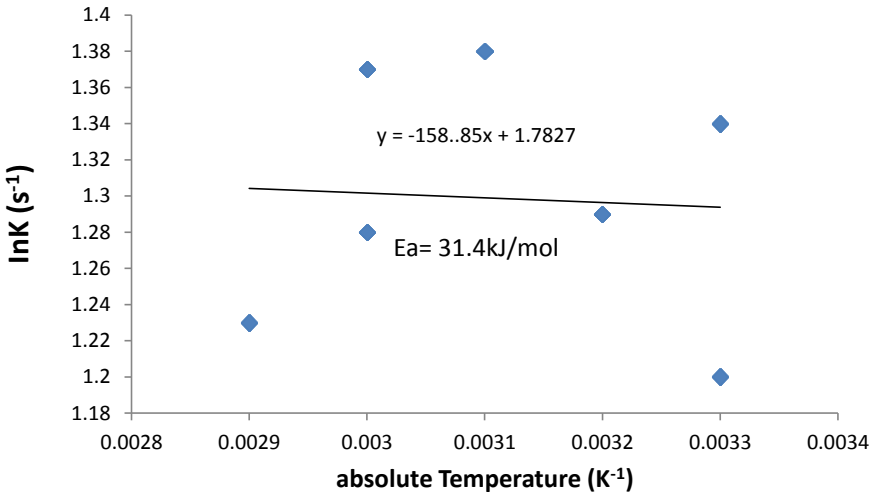

Figure 3. Arrhenius plot of human immobilized salivary α-amylase showing activation energy.

mechanism or may reflect an onset of denaturation of the enzyme (Sundarram and Murthy, 2014). A comparison of the optimum pH of the immobilized salivary a-amylase to those of microbial α-amylases showed that some

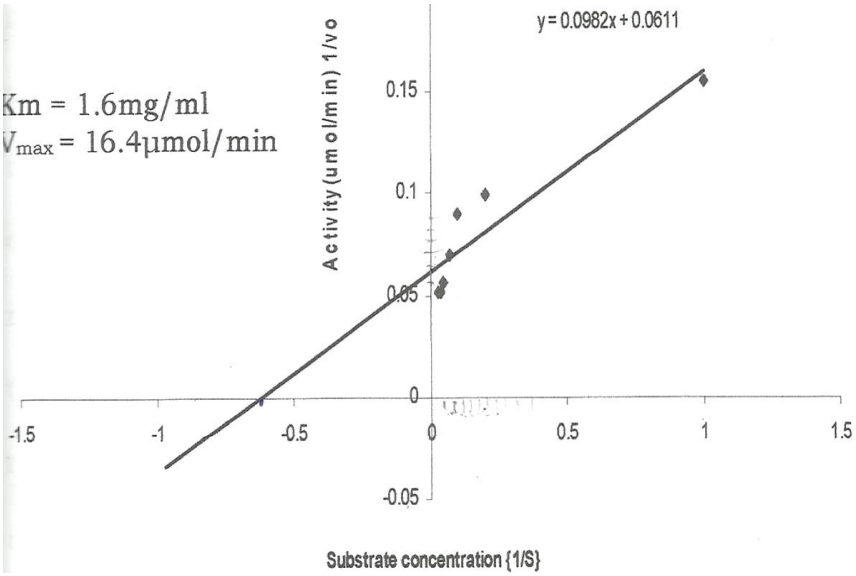

Figure 4. Line Weaver Burk plot of immobilized salivary α-amylase showing Km and Vmax values

Table 3. Comparison of Km values of immobilized salivary α-amylase and α-amylases from microbial sources.

Human/microbial species	Km (mg/ml)	Reference
Human saliva (immobilized)	1.6	This study
Bacillus sp. PB3224	2.7	Orlando et al. (1983)
B. subtilis	3.8	Ciobanu et al. (1976)
B. subtilis 65	1.2	Hayashida et al. (1988)
Bacillus sp. KCC103	1.9	Nagarajan et al. (2006)
truncated *Bacillus subtilis* alpha-amylase in *E. coli*	14	Marco et al. (1996)

microbial amylases are optimally active under acidic conditions, some under basic while others in a neutral medium. The activation energy (Ea), of the reaction was also investigated and found to be 31.4 kJ/ml (Figure 3). This is the minimum energy reactants, must possesses to overcome the activation barrier. The role of the enzyme is to reduce this barrier, thereby increasing the rate of reaction, hence the formation of products. From the Arrhenius plot, Ea is best regarded as the experimentally determine parameter that indicates the sensitivity of a reaction rate to temperature. Enzymes facilitate the rate of catalysis by providing an alternative route with less Ea for reacting species. Hence, Ea (31.4 kJ/mol) in this study is the energy required to hydrolyse starch to glucose molecules. More energy will have been required if the reaction was devoid of an enzyme.

The kinetic parameters Km and Vmax of the immobilized enzyme were obtained from a Lineweaver-Burk plot using starch as the substrate. The Km and Vmax were 1.6 mg/ml and 16.4 µmol/min, respectively. The value of the immobilized enzyme Km was lower than Km values from microbial sources (Table 3). The Km of an enzyme, relative to the concentration of its substrate under normal conditions permits prediction of whether or not the rate of formation of product, will be affected by the availability of the substrate. Since, Km is the major factor determining the enzymes' affinity for its substrate, which also affect the rate at which the enzyme is saturated by it substrate (Das et al., 2011) an enzyme with low Km has high affinity for its substrate and vice versa.

In conclusion, from the results obtained from immobilization of α-amylase from human saliva compared to that from microbial sources, immobilized salivary α-amylase is not thermally stable and may not be active in an acidic or basic medium though it has a low Km, signifying that the enzyme may have high affinity for its substrate starch which is of significant advantage to the industrial processes. Most industrial processes operate at very high temperature and during bioprocesses lot of acid and basic metabolites are released that alters the pH of

the bioreactor which may result to loss of enzyme activity. However, amylases from some microbial sources can operate in both acidic and basic medium and thus immobilized salivary α-amylase may not be a perfect alternative for industrial purposes.

Conflict of interests

The authors did not declare any conflict of interest.

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